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Initial study of thiocyanate microbial degradation by isolates from polluted soil in gold mining area in Indonesia

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A B S T R A C T

This study was conducted to clarify the ability of denitrifying bacterial group utilized nitrogen (N) due to their ability to decompose N in thiocyanate structure. Thiocyanate is a chemical substance that categorized as a pollutant in the environment, this chemical mainly generated by some industrial activities. Denitrifying bacterial group obtained from bulk of sludge samples collected from the gold tailing, and some soil samples collected around the gold mining site. The samples were taken to the Microbiology Laboratory, Research Center for Biology, to be investigated. Samples were initially acclimatized by potassium nitrate (KNO_3), acetonitrile, and liquid waste or sludge. The result showed that denitrifying bacteria in the samples could utilize 60 to 90% of NO_3-N (nitrate) in 42 days incubation. Isolation process were then conducted in each samples, and four denitrification bacterial, named as AN, Ea, L7T5, and PETI-7 isolates were obtained. The isolates formerly were cultured in a denitrifying bacterial medium containing KSCN (Potassium Thiocyanate), amended with glucose and sodium acetate as carbon source. Those four isolates performed satisfactory in aerobic and anaerobic cultures medium for denitrifying process, and utilizing glucose and sodium acetate as co-carbon source, but all bacterial isolates were unable to use thiocyanate as a single carbon source. Thiocyanate degradation performed by the isolates through a simultaneous conversion along with denitrification process. This phenomenon turn to open the opportunity on role of application denitrifying bacteria become bioresources material in efforts to decompose thiocyanate.

1. INTRODUCTION

Thiocyanate ($N{\equiv}C{-}S^-$), is a chemical compound consist of sulfur and single carbon element (C) which is banded with nitrogen (N), and naturally is a simple nitrile compound. Thiocyanate certainly formed through a cyanide detoxification process. On the other hand, thiocyanate also produced in the metal processing industry activities and the soda beverage manufacturer (Wood, 1975; Kelly & Baker, 1990). Thiocyanate is less toxic than cyanide but more stable and thus more difficult to be degraded. Various research has been successfully done using chemical and biological technologies. Furthermore, the research should be improved to understand thiocyanate metabolism and scale up technologies for degradation mode from laboratory to full-scale mode (Gould et al., 2012). Challenge arise in environmental issues because about 80 percent of gold production activities used cyanide to

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produce 2500 tons of gold per year to fulfill the world market need (KSDEA, 2000).

Thiocyanate is an intermediate compound in the cyanide biodegradation process, restraint by bacterial action. *Neutrophilic thiobacilli* bacteria known to use thiocyanate as electron donor for hovering energy and CO_2 fixation process (Youatt, 1954; De Kruyff et al., 1957; Happold et al., 1954; Happold et al.,1958; Katayama and Kuraishi, 1978; Smith and Kelly, 1988). Based on research done by Sorokin et al (2001) stated that oxidizing thiocyanate bacteria can grow chemolitotrophycly in high acidic and alkaline media. In the further study, that organism group comprised to genus *Thialkalivibrio*, which play role as the sulfur oxidizing bacteria and able to degrade thiocyanate (Sorokin et al., 2002).

Research in thiocyanate waste management based on oxidizing thiocyanate bacterial utilization has not been completed. In the early publication, de Kruyff et al (1957) described that *Thiobacillus denitrificans* able to grow in aerobic and anaerobic culture to exploit thiocyanate due to nitrate function as electron acceptor, and completely undertake nitrate reduction to become N_2. Some of research work also inform that *thiocyanate-dependent denitrification* happen because of multispecies bacterial population (*consortium*) commonly used in sewage treatment systems (Andreoni et al., 1988). In the other study, bacterial species of *Thiobacillus thioparus* only reduce nitrate to nitrite because of thiocyanate present in the media at aerobic process. Thiocyanate as liquid waste was usually reduced through activated-sludge process that have content of certain microbes to degrade thiocyanate as source of nitrogen or sulfur for their metabolic action. Broman et al (2017) worked to remediate wastewater containing metal sulfide ore, and found microbial consortium populations aligning within *Flavobacterium*, *Thiobacillus*, and *Comamonadaceae* lineages.

Considering to the fact that thiocyanate function is not only a source of nitrogen and sulfur, but also has role as electron donor for some certain bacterial group, so in this physiological study, we focused on denitrifying bacteria potential to metabolize thiocyanate. Four denitrifying bacteria were successfully isolated from sludge sample, and soil sample collected from traditional gold mining area. Bacterial growth and its denitrifying progress in associated to thiocyanate decomposing capacity were verified in this study. Mostly, gold mining activities produce thiocyanate because of cyanide application. Face the fact in the thiocyanate waste, the purpose of this research might become affordable to utilize denitrifying bacteria as bioresources agent for thiocyanate removal.

2. METHODS

2.1 Materials

Denitrifying bacterial isolates were taken out from 1.) Sludge sample collected from tailing pond in PT ANTAM, Pongkor, Bogor District, West-Java, Indonesia; 2.) Soil sample around tailing pond; 3.) A soil sample from traditional mining area in Cikotok, Banten Province. All samples were analyzed in Ecology Laboratory, Microbiology Division, Research Center for Biology, Indonesian Institute of Sciences. The isolation processes obtained four bacterial numbers and used in this study (Table 1).

2.2 Procedure

Liquid media for bacteria culture were prepared with 5 g Na_2CO_3; 2.5 g $NaHCO_3$; 1.25 g NaCl; 1 g K_2HPO_4; 100 mg $MgCl_2.6H_2O$; and 1.0 ml *trace element*. All the materials dissolved in 1000 mL aquadest, with pH 9.9. The media was autoclaved, in the temperature 121^{O}C, at 1 atm pressure, for 60 minutes. Augmented media such as Potassium Thiocyanate (KSCN), Potassium Nitrate (KNO_3), and Glucose were added at 500 -1000 mg, 1000 mg, and 2500 mg per liter media, respectively.

Denitrifying activities determined in liquid culture bacterial growth media by measuring NO_3^-N (nitrate) reduction, NO_2-N (nitrite) increase, KSCN (Potassium Thiocyanate) decline, and NH_4-N (ammonium) raise. Bacterial population measured with 436 nm optical density by spectrophotometric instrument (Carvalhal et al. 1991). Concentrations of ammonium, nitrate, nitrite, and thiocyanate were determined by Greenberg et al. (1992)

modified method. Chemical compounds were assessed through supernatant of liquid culture preparations.

Selected isolates (AN, Ea, L7E5, dan PETI-7) were tested in the followed media: **Media-1** contained liquid denitrifying bacteria and glucose; **Media-2** contained liquid denitrifying bacteria and thiocyanate (KSCN); **Media-3** contain liquid denitrifying bacteria and added with glucose and thiocyanate. One mL of isolates culture added with 9 mL media and placed in 300 mL- Erlenmeyer flask. Nitrogen (N_2) gas was flowed into the flask, 30 minutes, and the flask sealed tightly to make it anaerobic. The culture flask was shaken in the shaker with 100rpm and 7 days of incubation. The growth of denitrifying bacteria was checked daily by spectrophotometer instrument. Aerobic denitrification work was set at the same process, but without gas streamed into the flask.

Further verification of AN and Ea isolates was done to confirm the role of bacteria in degrading thiocyanates. Modified denitrifying media with KSCN, glucose, and sodium acetate (CH_3COONa) as the C source augmentation was intended to stimulate the denitrification process by bacteria. The incubation period was conducted at 20 days observation. Biological-cyanide Removal Efficiency (BRE) by bacterial isolates was calculated according to Mekuto et al (2016).

3. RESULT AND DISCUSSION

3.1 Acclimatization process

Dealing with denitrifying bacteria which is being capable to utilize thiocyanate as carbon sources (electron donors) (**Table 1**), therefore in the first step bacterial acclimatization was needed for cultures. Microbes were grown in liquid denitrification media, enriched with some organic carbon sources such as acetonitrile, acetic acid, and wastewater that were collected from tailing pond in gold mining activity. Through 42 days incubation, there was nitrate decreasing of nitrate content in the media culture with had varied decline grades at each sample source.

Decreasing of nitrate in media culture indicates that the existence of the denitrifying process was intent by microbes. Among each acclimatized culture of all samples, four denitrifying bacteria were obtained namely AN, Ea, L7T5, and PETI-7. The four bacterial isolates were tested for denitrification activity with carbon source of glucose and thiocyanate inserted in the media. The results showed that glucose renders as the carbon source and able to stimulate bacterial population (AN, Ea, L7T5, and PETI-7 Isolates) in liquid culture. Growth and metabolic processes in Media-1 presented the same pattern as those fully-fledged in Media-3, even though thiocyanate was present in Media-3 (**Figure 1**). Metabolite product of each bacterial isolate produced ammonium and nitrate which tend to be the same result during the incubation process.

Thiocyanate was inoculated into culture medium (Media-2) and leads to lowest bacterial growth because it had not contain glucose. These results designated that denitrifying bacteria in this study has very low potential to use KSCN without co-carbon source. Each isolate showed the same growth patterns on their metabolic activity when cultured with the media contain thiocyanate and lack of glucose for microbial carbon source.

Table 1. Denitrifying bacteria isolated from sludge and soil samples

Sample Source	NO_3^-N decrease in the sample for 42 days incubation, acclimated with:	Denitrifying Verification:		Isolates Code
		Growth in the media containing KSCN	Gas Produce	
Gold tailing pond in PT ANTAM	Acetonitrile	AN Isolate	+	AN
	Sludge	Ea Isolate	+	Ea
Cikotok Soil	Sludge	L7T5 Isolate	+	L7T5
Pongkor Soil	Sludge	PETI-7 Isolate	+	PETI-7

3.2 Thiocyanate verification

Based on the individual growth, Ea Isolate behaves differently in ammonium change along its metabolic activity. On the other side, AN isolate performed the highest growth in media enriched by glucose. Referring the above mentioned, therefore further study to AN and Ea Isolates was continued. Both isolates were then tested by using sodium acetate (CH_3COONa) as carbon sources to proof their growth and denitrification activity, as well as its ability in thiocyanate degradation. Results of the studies were listed in **Table 2**. Those bacteria isolates were able to grow and utilize CH_3COONa as the carbon source. Increasing of microbial population and decreasing nitrate concentration in the culture media become indicator of denitrifying process and simultaneously due to thiocyanate metabolism by bacteria. Ea isolate has more absorption compared to AN isolate, this is proof that Thiocyanate was more reduced by Ea isolates, especially with CH_3COONa present in the culture. The data below confirm that denitrifying bacteria become less for its capability to use thiocyanate due to single carbon source in the media without co-carbon source.

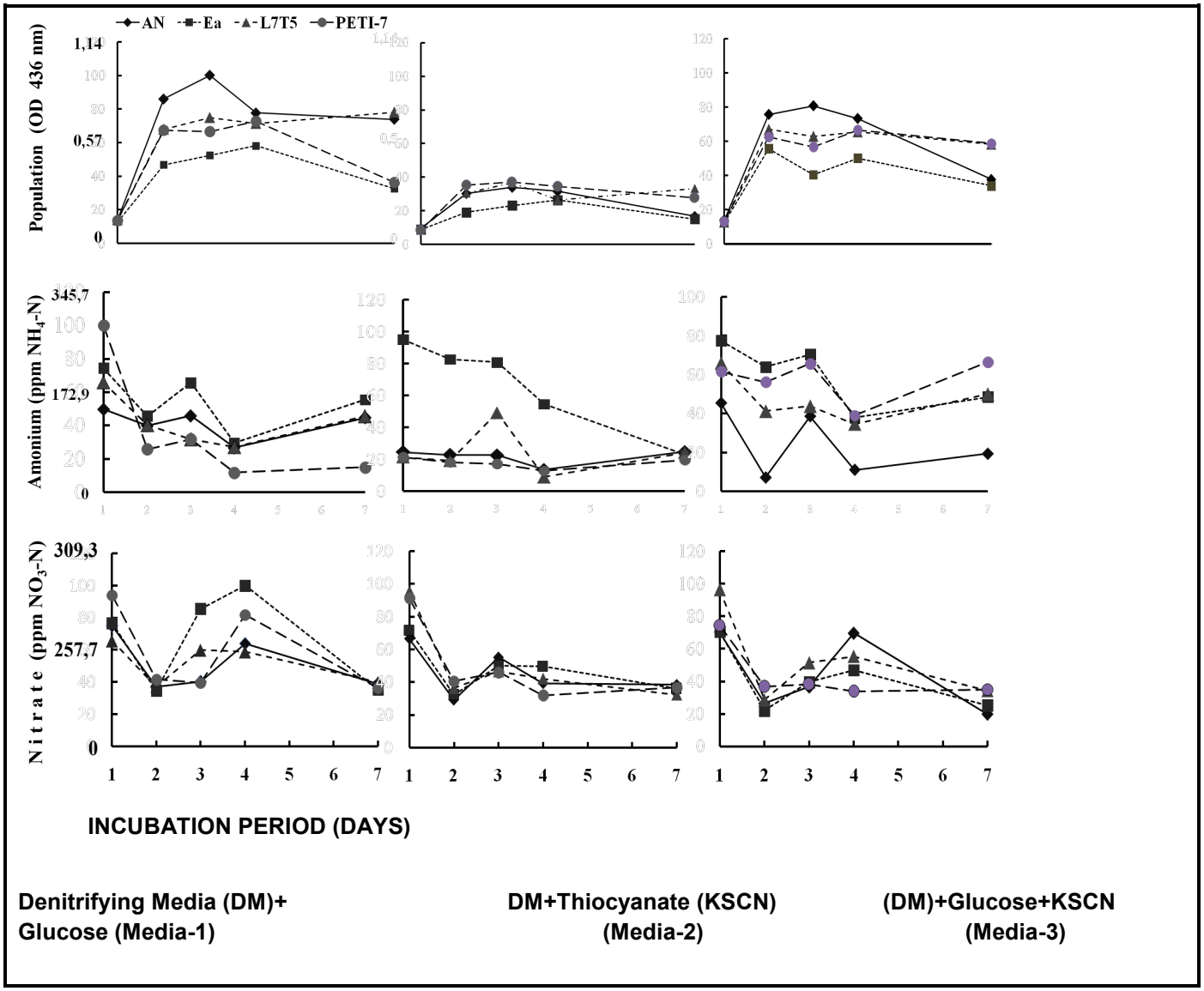

Figure 1. Growth, performance and N metabolic activity in anaerobic condition

Table 2. AN and Ea Isolates in denitrifying liquid media augmented by KSCN and Sodium Acetic Acid (CH3COONa) at twenty days incubation period

Parameters & incubation period		AN Isolate growth in: KSCN+CH_3COONa	Ea Isolate growth in: KSCN+CH_3COONa	Ea Isolate growth in: KSCN
Population (OD 436 nm)	3	0,066	0,160	0,072
	10	0,298	0,166	0,237
	20	1,432	0,585	0,366
Thiocyanate (ppm KSCN)	3	713,07	751,66	667,77
	10	708,75	639,12	642,44
	20	705,65	635,65	640,22
Ammonium (ppm NH_4-N)	3	5,80	10,34	7,80
	10	15,34	18,78	8,60
	20	23,95	0,00	26,75
Nitrate (ppm NO_3-N)	3	32,93	31,98	28,30
	10	5,67	1,60	24,60
	20	5,34	0,42	3,00
Nitrite (ppm NO_2-N)	3	0,551	0,949	0,130
	10	0,287	12,545	1,493
	20	1,135	14,106	3,455

As the complement of this study, denitrifying bacteria also assessed in an aerobic situation. Other studies had reported that denitrifying bacteria can grow both in anaerobically and aerobically (de Kruff et al., 1957: Andreoni et al., 1988). The results of aerobic culture were shown in **Figure 2**, which are indicating that bacterial growth utilizes thiocyanate.

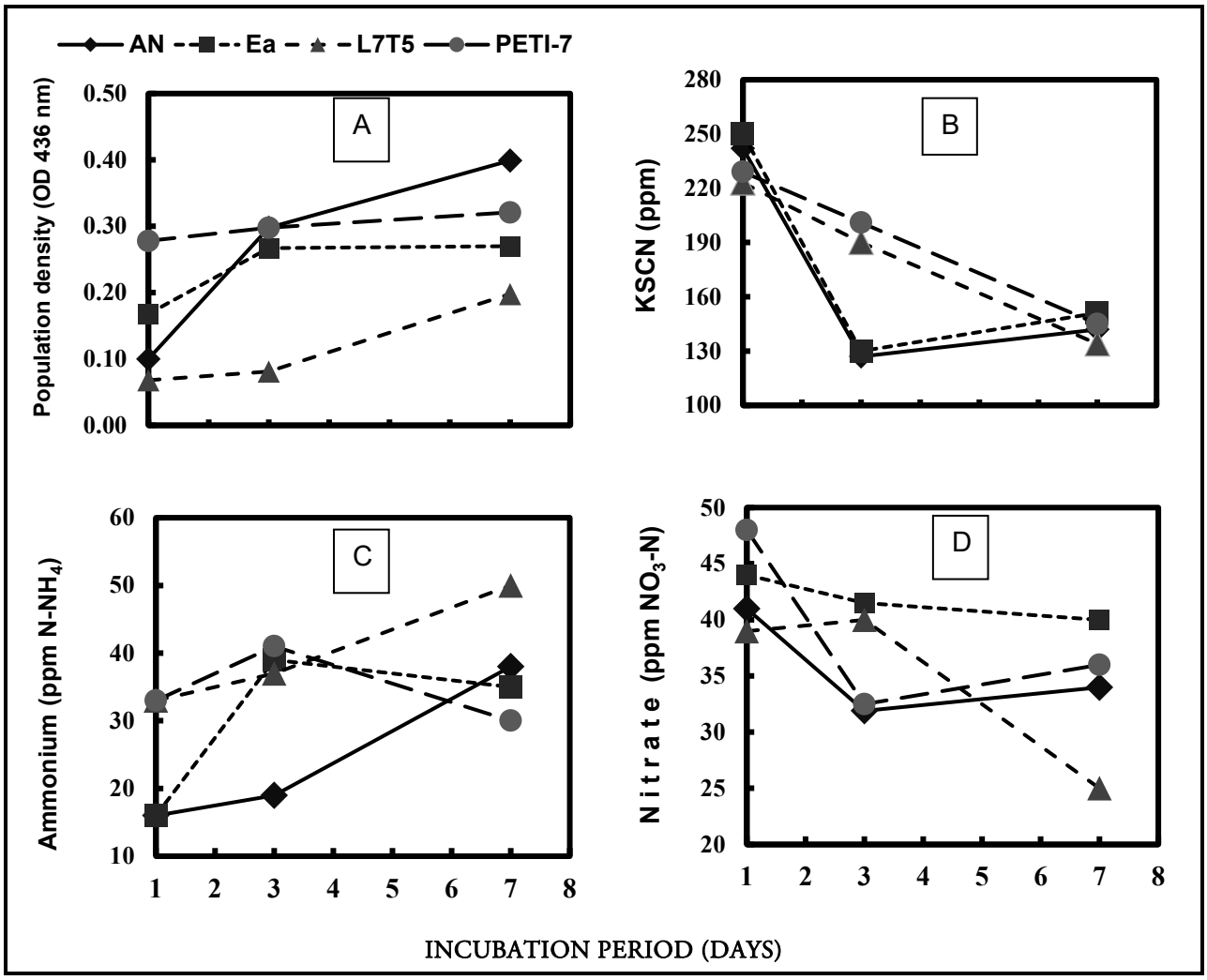

Figure 2. Growth pattern and metabolic activity of Isolates (AN, Ea, L7T5, and PETI-7) in an aerobic situation

Even if the growth tends to escalate in the 7-day incubation period (Figure 2A), denitrification activity was also had appropriate balance among nitrate decrease (Figure 2D) to ammonium increase (Figure 2C). Refer to the result shown that increasing of the bacterial population has opposite fact to nitrate and thiocyanate decline (Figure 2B) in the media. This phenomenon was designated strongly confirm that denitrifying bacteria had performed denitrification process simultaneously with thiocyanate degradation.

Correlation among parameters has been valued in **Table 3** and showed that AN and Ea Isolates are denitrifying bacteria which is capable to degrade thiocyanate. Bacterial population increased which is analogously become turns down of nitrate and thiocyanate content in the media. It means that the change of nitrate and thiocyanate might become strong indication that isolates are denitrifying bacteria which can reduce thiocyanate.

The presence of Ammonium (NH_4) accumulation was also strong signal of thiocyanate degradation due to denitrifying exertion. It has been reported that cyanate degradation reactions as due to microbial metabolism process is as follows: SCN- + 2 H_2O + 2½ O_2 ---> SO_4^{2-} + HCO_3^- + NH_3.

Denitrifying bacteria are heterotrophic and requiring organic carbon for an energy source, which play as an electron donor to perform denitrification reactions. In this situation, results of the study informed that four bacterial isolates growth and denitrification activity are inhibited

when the thiocyanate (KSCN) was used as a single carbon source, without the addition of glucose or sodium acetate. Growth inhibition occurred and it is signed to low growth in bacterial population (OD) when compared with glucose or sodium acetate added into media as carbon source (**Figure 1**).

Inhibition in the denitrification process was indicated by nitrate (NO_3) decrement, but denitrification more slothful due to sodium acetate absence in the media culture (**Table 2**). From this data could be concluded that the thiocyanate decrease was not because of used as energy source for bacterial growth, it was likely to be converted into other chemical compounds, and Budaev et al (2015) were mention in research exertion. Thiocyanate degradation was not observed in this study, but ammonium formation in media culture become virtuous evidence of degradation.

AN Isolate was increased in the population rate and correlated to ammonium accumulation (-Avs.C-) in the culture medium. The value among thiocyanate disappears to ammonium arise (-Bvs.C-), and also to nitrate decrease (-Bvs.D-) in the media were strongly correlated. Based on the data above, there was affirmation for denitrifying bacteria in the study which are able to degrade thiocyanate. Ea Isolates had almost the same metabolic pathway compared to AN performance, but Ea Isolate had tendentiously stronger to degrade thiocyanate. However, all of four isolates actually had almost similar ability to degrade thiocyanate around 3 to 8 percent in Biological Removal Efficiency (BRE) per 24 hour degradation (**Figure 3**).

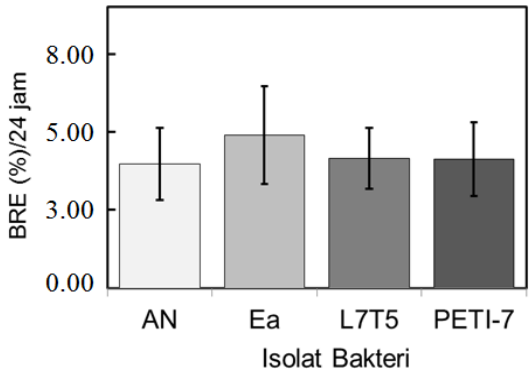

Figure 3. Biological Removal Efficiency (BRE) of Denitrifying Bacteria in the media contain 500 mg/L KSCN

Refer to other other research, thiocyanate commonly becomes energy source for chemolithotrophic sulfuric oxidizing bacteria. Thiocyanate was degraded through two different pathways response. The first reaction works by breaking C-S bonds to cyanate intermediate form ($N{\equiv}C{-}O^{-}$), and if any bicarbonate present then it turns into ammonia and CO_2 by cyanase enzyme response (Youatt, 1954; Happold et al., 1958). The second reaction was hydrolysis process of the nitrile bond ($N{\equiv}C$) which are forms of carbonyl sulfite (S=C=O) and ammonia (Katayama et al., 1992; Katayama et al., 1993; Katayama et al., 1998). Carbonyl sulfite hydrolyzed further into sulfites and CO_2. This sulfite can be utilized as a source of energy and electron donor by autotrophic bacteria for growth.

Table 3. Correlation among parameters based on AN and Ea Isolates in denitrifying liquid media containing KSCN and Sodium Acetic Acid (CH_3COONa)

Parameters (n-1= 2; $p_{0,10}$ & $p_{0,02}$ **r =** 0,900 & 0,980)	AN Isolate growth in: [KSCN+CH_3COONa]			Ea Isolate growth in: [KSCN+CH_3COONa]		[KSCN]
	-Avs^C-	-BvsC-	-BvsD-	-AvsC-	-BvsD-	-AvsB-
Population density - A -						
KSCN degradation - B -	0.923*	0.914*	0.914*	0.982**	0.998**	-0.929*
Ammonium increases - C -						
Nitrate decreases - D -						

* correlated; ** strongly correlated; ^vs. = versus

Three thiocyanate-oxidizing bacterial strains, *Burkholderia* sp., *Chryseobacterium* sp., and *Ralstonia* sp. were isolated by Huang et al (2013) from the activated sludge inside coke wastewater treatment plant. Bacterial biodegradation proceeded individually with the highest rate and utilize thiocyanate as sole carbon source at pH 7.7 and 35^{O}C. This study may provide design for large scale operation of aerobic bioreactor for thiocyanate treatment.

Thiocyanate is also established to become nitrogen sources for algae to produce lipid. Two cultivation methods were developed by Ryu et al (2014) throughout the consortium mode based on algae and bacteria. There are a lithoautotrophic and a photoautotrophic mode. Thiocyanate hydrolysis and a nitrification was occurred under the first (lithoautotrophic) condition, while the oxidized forms of nitrogen were assimilated by the photoautotrophic consortium, and lipids were produced under the second condition.

Usage of microbes for requiring biological degradation of thiocyanate from contaminated wastewater had been successful at the laboratory investigation by van Zyl et al (2015). In the further analysis revealed that the presence of solids could decrease microbial diversity; but it showed that many organisms have genes for denitrification and sulfur oxidation, otherwise the only one genus of *Thiobacillus* sp. present in the culture growth (Rahman et al., 2016). Nitrifying bacteria achieved from this work could be improved as above mode technique to improve application in bioremediation.

4. CONCLUSION

Results of this study showed that four denitrifying bacterial isolates can be alive for aerobic and anaerobic denitrification process. Those bacteria could use glucose and sodium acetate as trigger to convert thiocyanate but were unable to use thiocyanate as the single carbon source. Aerobic or anaerobic denitrification activities on both alive cultures could metabolize organic carbon sources of glucose and sodium acetate but were unable to exploit thiocyanate as the only single carbon source. Decreasing of thiocyanate in the media culture mostly performed by denitrifying bacterial growth through the process of degradation or conversion into certain compounds and ammonium. Denitrification process and thiocyanate degradation can occur simultaneously, both in aerobically and anaerobically conditions. Bacterial capacity to degrade thiocyanate is almost the same in all four bacterial isolates. Handling of thiocyanate waste can be manipulated by utilizing the denitrifying bacteria with carbon source induction. Manipulation of carbon sources can be pursued through the utilization of organic carbon sources that are widely available in nature such as cellulose, hemicellulose, lignocellulose and other sources as agricultural waste, and further research is needed.

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